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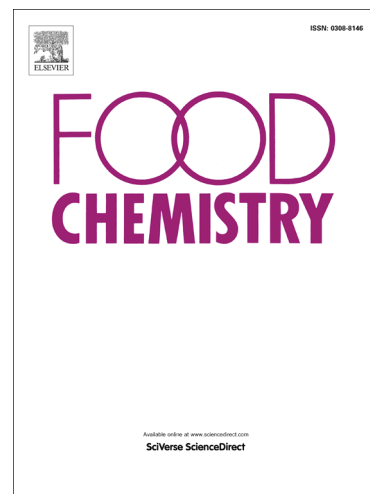
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**Effect of amaranth proteins on the RAS system. In vitro, in vivo and ex vivo  
assays**

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**Abstract**

The aim of this work was to analyse the hypotensive effect of amaranth protein/peptides on spontaneously hypertensive rats (SHR). The mechanism of action of these peptides was studied *in vivo* and *ex vivo*. We also tested the effect of protection against gastrointestinal digestion (GID) exerted by an O:W emulsion on the integrity of the antihypertensive peptides. All samples tested produced a decrease in blood pressure (SBP). The animals treated with emulsion ( $G_E$ ) and emulsion+peptide ( $G_{E+VIKP}$ ) showed the most significant reduction in the SBP ( $42 \pm 2$  mmHg and  $35 \pm 2$  mmHg, respectively). The results presented suggest that after GID, a variety of peptides with biological activities were released or were resistant to this process. These peptides play a role in the regulation of the SBP by acting on plasma ACE, plasma renin and the vascular system. These results support the use of amaranth protein/peptides in the elaboration of functional foods for hypertensive individuals.

**Key words:** amaranth proteins, antihypertensive activity, protein hydrolysate, spontaneously hypertensive rats, renin-angiotensin system.

## 1 Introduction

Hypertension is one of the risk factors associated with cardiovascular disease. This condition can be managed through regular physical activity, a healthy diet, a reduced sodium intake, and avoiding smoking, among others (Norris & FitzGerald, 2013; WHO, 2013). Blood pressure is known to be regulated through a complex mechanism involving several interconnected metabolic pathways. Hypertension is characterised by an increased activity of the enzymes (mainly renin and angiotensin converting enzyme, ACE) belonging to the renin-angiotensin system (RAS). The activation of this system leads to high levels of angiotensin II, which causes blood vessels to contract, together with a reduction in vasorelaxation and an increase in the production of superoxide anion. Together, these phenomena lead to an increased NO degradation rate with the consequent decrease in its vasorelaxant effect (Aluko, 2015a; Norris & FitzGerald, 2013). The RAS also comprises ACE2 and the peptides angiotensin (1-7) and angiotensin (1-9), which have opposite effects to angiotensinogen II (Nguyen Dinh Cat & Touyz, 2011).

The European guidelines for the management of hypertension indicate that functional foods might be a good alternative for patients with mild hypertension or act as a co-adjuvant of anti-hypertensive drugs in the treatment of moderate hypertension (Borghi, Urso, & Cicero, 2017). Functional foods, which can be used for either the treatment or the prevention of hypertension, do not cause the adverse effects of conventional drugs. The employment of these foods may contribute to the reduction of the public health expenditure destined to the cure of cardiovascular diseases (Houston, 2019a, 2019b)

Bioactive peptides of animal and vegetal origin, as well as some components of functional foods, exert anti-hypertensive effects mainly through the inhibition of RAS enzymes and the increase of NO levels (Aluko, 2015a, 2015b, 2019; Fritz, Vecchi, Rinaldi, & Añón, 2011; He, Aluko, & Ju, 2014; Hernández-Ledesma, Del Mar

Contreras, & Recio, 2011; Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012; Quiroga, Aphalo, Nardo, & Añón, 2017; Saleh, Zhang, & Shen, 2014; Wu, Liao, & Udenigwe, 2017).

Amaranth proteins are a source of bioactive peptides. Over the last years, these proteins have gained an interest that goes beyond their nutritional properties. In fact, many compounds with beneficial effects on health have been characterised, particularly, the biologically active peptides obtained through enzymatic hydrolysis of proteins from this pseudocereal (Tovar-Pérez, Lugo-Radillo, & Aguilera-Aguirre, 2018). Studies carried out in our laboratory have demonstrated the existence of cryptic peptides in reservoir proteins of amaranth grains. These peptides have proved to inhibit ACE and renin (Quiroga et al., 2017; Quiroga, Aphalo, Ventureira, Martínez, & Añón, 2012; Tiengo, Faria, & Netto, 2009; E. G. Tovar-Pérez, Guerrero-Legarreta, Farrés-González, & Soriano-Santos, 2009; Vecchi & Añón, 2009; Vilcacundo, Martínez-Villaluenga, Miralles, & Hernández-Ledesma, 2018). Furthermore, our research group is the first one to have performed *in vivo* experiments using an amaranth hydrolysate. This preparation was found to reduce the blood pressure values (Fritz et al., 2011).

Thus, amaranth proteins have cryptic peptides with anti-hypertensive properties demonstrated both *in vivo* and *in vitro*. Based on the results reported so far, we consider that amaranth peptides act as hypotensive bioactive peptides through different mechanisms. However, the mechanism governing their anti-hypertensive effect has not been elucidated. The aim of this work was, therefore, to perform further research on the biological effect of amaranth peptides. To that end, the hypotensive effect of samples containing amaranth protein/peptides was tested on spontaneously hypertensive rats (SHR). The mechanism of action of these peptides was studied both *in vivo* and *ex vivo* by evaluating the quantity and the biological activity of the main RAS enzymes (ACE and renin). Amaranth samples were also tested on vascular endothelium. We also tested the effect of protection against gastrointestinal digestion

exerted by an O:W emulsion on the integrity of the antihypertensive peptides present in an amaranth hydrolyzate.

The results presented in this work will allow the development of strategies for the incorporation of these anti-hypertensive peptides into functional foods.

## 2 Materials and Methods

### 2.1 Chemicals

The Ratrenin ELISA kit MBS041519 and the Rat angiotensin converting enzyme ELISA kits MBS703086 (MyBioSource, San Diego, CA, USA) were employed to assess the activity of renin and ACE. All other reagents were purchased from Sigma (St. Louis, Missouri, USA).

### 2.2 Samples

The following samples were used for *in vivo* assays:

- Amaranth protein isolate (API) and hydrolysate (AH) prepared from *Amaranthus hypochondriacus* as described elsewhere (Suárez & Añón, 2019). The protein content was  $87 \pm 1$  and  $57 \pm 2$  % w/w w.b for API and AH respectively.
- VIKP peptide, which is a synthetic peptide from the 11S amaranth protein. This peptide has inhibitory activity on ACE (Vecchi & Añón, 2009).
- O/W 20:80 emulsions prepared with sunflower oil and 1:1 protein mixtures of API and AH at pH 2 and with a total protein concentration of 2% with or without the VIKP peptide [(API50+AH50)-2%+VIKP and (API50+AH50)-2%, respectively]. Emulsions were prepared according to Suárez & Añón, 2019. Emulsions were frozen at  $-80^{\circ}\text{C}$ , lyophilised and resuspended as required. Before administration, the resuspended emulsions were homogenised with a magnetic stirring bar.
- Commercial ACE and renin inhibitors (captopril and aliskiren, respectively) were employed as positive controls.

### 2.3 *In vivo* assays

#### 2.3.1 Animals

*In vivo* and *ex vivo* experiments were carried out at the Cardiovascular Laboratory of the Chair “A” of Histology, Embryology and Cytology, School of Medical Sciences, National University of La Plata (UNLP). The protocol for animal handling was approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL), protocol number P02-05-2017, School of Medical Sciences-UNLP. Sixty male SHR rats were acquired at the Experimentation Animals Laboratory (LAE) of the School of Veterinary Sciences, UNLP. The animals weighed between 210-290 g and had approximately 10 weeks of age at the beginning of the study.

Rats were housed in stainless steel cages, 4 animals per cage, with sterilised bedding (white pine wood shavings), which was changed every day. The facility had air conditioning and a 12 h light -12 h dark cycle. The environmental humidity was kept at optimum levels for the experiment and animal well-being. Food and water was provided *ad libitum*. Tap water was provided in sterilised bottles with stainless steel nipples. Animals were fed with extruded balanced feed (rat-mouse, Ganave, Grupo Pilar S.A (Buenos Aires, Argentina).

### **2.3.2 Oral administration of samples**

Samples indicated in 2.2 were administered to different groups of SHR rats as follows:

1. Negative control group (water,  $G_W$ ): animals which did not receive amaranth protein.
2. Captopril group ( $G_C$ ): animals treated with captopril, an ACE inhibitor.
3. Aliskiren group ( $G_A$ ): animals treated with aliskiren, a renin inhibitor.
4. API group ( $G_{API}$ ): animals treated with API.
5. AH group ( $G_{AH}$ ): animals treated with AH.
6. VIKP group ( $G_{VIKP}$ ): animals treated with the synthetic *in vitro* anti-hypertensive peptide.
7. Emulsion group ( $G_E$ ): animals treated with API50+AH50 2% pH 2.
8. Emulsion + VIKP group ( $G_{E+VIKP}$ ): animals treated with (API50+AH50)-2%+VIKP.

Samples were administered by the orogastric route with 2 ml of each sample dispersed in distilled water. The  $G_w$  group received 2 ml of distilled water, groups  $G_C$  and  $G_A$  received 10 mg captopril/kg and 2.5 mg aliskiren/kg, whereas groups  $G_{API}$ ,  $G_{AH}$  and  $G_E$  received 1.9 g protein/kg, group  $G_{VIKP}$ , 50 mg peptide/kg, and group  $G_{E+VIKP}$ , 1.9 g protein + 50 mg peptide. Animals were divided into 8 groups of 7-8 animals each, except captopril and aliskirene groups in which 4 animals were used. The average starting SBP corresponding to 58 animals was  $191 \pm 22$  mmHg.

### **Indirect measurement of blood pressure**

Rats were placed in a chamber at  $37^\circ\text{C}$  for 10 min, and then transferred to a standard setup (Fritz & Rinaldi, 2007) with heating pad and an acrylic restrainer, a tail cuff and a pulse sensor (NarcoBiosystems, Houston, TX). The systolic blood pressure was measured according to Fritz et al., (2011).

In order to determine baseline values, blood pressure values were recorded at least three times in different days for each rat.

After recording the last baseline blood pressure value, an aqueous suspension of each sample was administered to each animal. Three hours after the administration, blood pressure values were recorded.

### **2.3.3 Surgical procedure**

Animals were anesthetized with an intra-peritoneal injection (23 gauge needles) of sodium pentobarbital (25 mg/ml, 0.15 ml/100 g) and diazepam (0.25 mg/ml, 0.1 ml/100 g). The rat's abdominal aorta was then cannulated to collect a blood sample (roughly 6 ml) in heparin-coated tubes. Samples were immediately centrifuged at  $4^\circ\text{C}$  for 15 min and 1000  $\times g$ . The plasma was separated, aliquoted and kept at  $-80^\circ\text{C}$  until used.

### **2.3.4 Determination of plasma ACE and renin concentrations**

A commercial ELISA kit (Ratangiotensin converting enzyme MBS703086, MyBioSource, CA, USA) was employed to determine the concentration of ACE according to the manufacturer's directions. This immunoassay is based on a



competitive inhibition. Briefly, microtitre plates are coated with ACE. Samples and standards are incubated together with an anti-ACE HRP-labeled conjugate to generate the competition.

The plasma renin concentration was determined with a commercial ELISA kit (Rat renin ELISA kit MBS041519 My BioSource) following the manufacturer's directions. This immunoassay is a direct ELISA, which has an analytical measurement range of 6.25 pg/ml - 200 pg/ml. The final colour reaction was read in a microtitre plate reader (Biotek Synergy HT, Winooski, VT, USA) at 450 nm.

### **2.3.5 Determination of plasma ACE activities**

ACE-inhibitory activity was assayed according to Suárez & Añón, (2019). Briefly, to determine the enzymatic activity, 50 µl of buffer [0.2 M sodium borate pH 8.3; 2 M NaCl), 25 µl of mili Q water, 25 µl of the commercial enzyme (maximum activity control)], or plasma samples were incubated with 100 µl of synthetic substrate (HHL) at 37°C for 30 min. The reaction was stopped by heating the mixture over a water bath at 90°C for 15 min. After cooling, 600 µl of 0.2 M potassium pH 8.2 and 515 µl of colour reagent, which reacts with the hippuric acid generated during the enzymatic reaction, were added and stirred vigorously with a vortex and then centrifuged for 10 min at 20°C and 3000 *xg*. The absorbance was measured at 382 nm in a spectrophotometer (Beckman DU 650).

The reaction blank was obtained by incubating the synthetic substrate with neither the plasma samples nor the enzyme, completing the reaction volume with mili Q water.

Reaction blanks without the substrate (the HHL was replaced with 100 µl of borate buffer) and containing plasma samples were also included. Controls containing plasma samples and captopril were also assayed.

### **2.4 Ex vivo experiments**

During the surgical procedure employed to obtain blood samples, the thoracic aorta was resected and placed in saline bubbled with 5% CO<sub>2</sub> and 95% O<sub>2</sub>. The

adjacent connective tissue was carefully removed avoiding distention of the vessel and damage to the endothelium. The aorta was then cut into two mm long rings. The assay was performed according to Fritz et al., (2011). Briefly, the smooth muscle tissue was stabilised in a chamber for 1 h with a resting tension of 2-3 g, with changes of saline every 20 min. Tissue rings were then exposed to a solution containing 80 mM potassium or increasing concentrations of norepinephrine ( $10^{-6}$  and  $10^{-7}$  M). For each condition, the contractile response was recorded. At the end of the experiment, tissue rings were dried on filter paper and weighed on a precision scale. The contraction intensity was calculated as the quotient between strength and the weight of the ring (mgF/mg).

## 2.5 Statistical analysis

The statistical analysis was performed with the GraphPad Prism 6.0 (GraphPad Prism Inc., CA, USA). The results obtained in *in vivo* experiments were expressed as means  $\pm$  standard error of the mean (SEM). In order to compare mean values, a one way analysis of variance (ANOVA) multiple comparisons was applied. The critical significance level was set at  $p < 0.05$ . The Least Significant Difference (LSD) test was used to analyse the results obtained *in vivo*.

## 3 Results

### 3.1 Effect of the isolate, hydrolysate and emulsions containing amaranth peptides

The hypertensive rat is a suitable model to study systemic hypertension in humans caused by an increased production of angiotensin II produced by the action of ACE and renin. AH, O/W emulsions, VIKP and captopril presented inhibitory ACE activity *in vitro*, whereas AH and aliskiren inhibited renin *in vitro* (**Table 1**). The inhibitory activity of API was very low for both ACE and renin enzyme (**Table 1**). All samples, except  $C_w$ , caused a reduction of different magnitudes on the baseline systolic blood pressure (SBP), which was  $19 \pm 3$  mmHg on average. **Figure 1** shows the reduction in the SBP values exerted in each experimental group. Values were

expressed as the decrease of SBP in mmHg of animals 3 h after the administration of each sample with respect to the SBP measured at the beginning of the experiment ( $SBP_{0hi} - SBP_{3h}$ ).

The decrease of SBP in the  $G_w$  group was  $3 \pm 8$  mm Hg. The high dispersion observed in this parameter was due to an inter-individual variability presented by this experimental group. In animals treated with commercial anti-hypertensive drugs, the reduction in the SBP was  $32 \pm 4$  mmHg and  $45 \pm 5$  mmHg, for  $G_C$  and  $G_A$  groups, respectively. These animals reached blood pressure values somewhat higher than that corresponding to normotensive rats ( $\sim 128$  mmHg) (Li et al., 2017, 2016); it is worth remembering that the treatment carried out with the samples under study was one dose treatment of 3 hours. The results obtained with  $G_C$  and  $G_A$  group are not surprising considering the localisation and action of renin and ACE in the RAS since renin is the rate-determining enzyme in the RAS system.

Animals belonging to the  $G_E$  and  $G_{E+VIKP}$  groups showed the most significant reduction in the SBP, reaching reduction values of  $42 \pm 2$  mm Hg and  $35 \pm 2$  mm Hg, respectively. The administration of API, AH or VIKP in water as vehicle ( $G_{API}$ ,  $G_{AH}$  y  $G_{VIKP}$  groups) caused a reduction in SBP values that was significantly lower than those observed in the groups mentioned above ( $25 \pm 14$  mmHg,  $26 \pm 3$  mmHg and  $21 \pm 3$  mmHg, respectively.)

Our research group has recently demonstrated that an emulsion formulated with a mixture of amaranth protein isolate and hydrolysate (API + AH) can inhibit ACE *in vitro* ( $IC_{50}$   $0.29 \pm 0.03$  mg/ml). Besides, an artificial digestion process does not abrogate the inhibitory capacity of the emulsion on ACE ( $IC_{50}$   $0.13 \pm 0.07$  mg/ml (Suárez & Añón, 2019). These results strongly support the idea that either the anti-hypertensive peptides present in the emulsion are resistant to proteolysis and/or that during the digestion process anti-hypertensive peptides are generated. These findings are in line with those of Tiengo et al., (2009), who demonstrated that protein hydrolysates from *A. cruentus* concentrates obtained with alcalase did not increase

their inhibitory activity on ACE after gastrointestinal digestion, as compared with the undigested hydrolysate. The authors concluded that the peptides released by alcalase were resistant to the hydrolytic effect of digestive enzymes.

A few studies have demonstrated the reduction of the SBP in SHR rats by the administration of amaranth protein/peptides or other protein sources. Fritz et al., (2011) have demonstrated a 25 mmHg decrease in the SBP after 1.5 h post-administration of amaranth hydrolysates, with such effect lasting for 7 h. Working with hydrolysates of genetically modified amaranth 11S globulin (Medina-Godoy et al., 2013), and with amaranth protein hydrolysates obtained with alcalase (Ramírez-Torres et al., 2017), achieved reductions of blood pressure values of 20-25 mmHg and 28 mmHg, respectively, after 4 and 3 h post-administration, respectively. Hypotensive effects have also been achieved with other protein sources, such as bovine seroalbumin peptides (Lafarga, Rai, Connor, & Hayes, 2016), cod (Girgih et al., 2015), gelatin (Ngo et al., 2015), and rice proteins (Li, Qu, Wan, & You, 2007).

To date, the present work and that of Fritz et al. (2011) and Ramírez-Torres et al. (2017) are the only ones evaluating the effect of amaranth hydrolysates prepared with alcalase on SHR rats.

### 3.2 Effects on plasma renin and ACE

In order to assess the effect on the levels of RAS enzymes of each preparation administered orally, the concentration of ACE and renin were determined in each experimental group. Only the  $G_A$ ,  $G_{API}$  and  $G_{AH}$  groups presented differences in the plasma renin levels, as compared to the  $G_W$  group (**Figure 2 A**). No differences were found in the levels of this enzyme between  $G_C$ ,  $G_E$ ,  $G_{VIKP}$  and  $G_{E+VIKP}$  groups and the control group  $G_W$ .

A different behaviour was observed for the plasma levels of ACE. In **Figure 2, B**, it can be observed that the  $G_W$  group presented extremely low values of ACE ( $0.17 \pm 0.02 \mu\text{g/ml}$ ), whereas the concentration of ACE in  $G_C$ ,  $G_A$ ,  $G_E$  y  $G_{E+VIKP}$  groups was 13.6 to 25.8 times higher than that of the control group. The administration of the

isolate, the hydrolysate, and the peptide ( $G_{API}$ ,  $G_{AH}$ ,  $G_{VIKP}$  groups, respectively) induced an increase in the ACE levels that was 7.6 to 5.3 times higher, as compared to the control group ( $1.3 \pm 0.2 \mu\text{g/ml}$ ,  $0.90 \pm 0.3 \mu\text{g/ml}$  and  $1.1 \pm 0.3 \mu\text{g/ml}$ , respectively,  $p < 0.05$ ). However, this increase was of a lower magnitude than that exerted by captopril, aliskiren and the emulsion with or without the peptide.

Studies evaluating synthetic drugs for the treatment of hypertension have also demonstrated an increase in the levels of ACE in plasma and tissues. Chai, Perich, Jackson, Mendelsohn, & Johnston (1992) have found that lisinopril administered chronically (14 days, 10 mg/kg) to Sprague-Dawley rats induced a significant increase in ACE levels in plasma and lung tissue, but not in the aorta, testicles or kidneys. On the other hand, Masahairo et al., (1991) have found that the administration of the same drug chronically to the same strain of rats induced a 1.75-fold increase in the total plasma levels of ACE and of a 30% increase of the ACE levels in lungs, as compared to controls. These authors did not find any change in the levels of this enzyme in neither the kidneys, nor the adrenal glands, nor the aorta. In that work, it was also found that during the treatment, the ACE activity in these organs could be inhibited with low levels of free ACE.

The increase of ACE in plasma and lungs does not seem to decrease the efficacy of ACE inhibitors in patients with chronic hypertension or chronic heart failure (Chai et al., 1992).

The activity of ACE in plasma was then determined in order to assess whether the increased levels of this enzyme after the administration of conventional ACE and renin inhibitors and the API + AH emulsion formulated with or without VIKP has any biological relevance. As shown in **Figure 3**, the ACE activity was found to be inversely proportional to its plasma concentration. The lowest activity values (4-7 % active ACE/ $\mu\text{g}$  plasma ACE) corresponded to groups  $G_C$ ,  $G_A$ ,  $G_E$  and  $G_{E+VIKP}$ , whereas the highest activity values were found in the  $G_{VIKP}$ ,  $G_{AH}$  and  $G_{API}$  groups, which presented 20-13% active ACE/ $\mu\text{g}$  plasma ACE.

Taken together, these results would indicate that the action of ACE competitive inhibitors, such as captopril or the hypotensive amaranth peptides either present in the samples or generated during the gastrointestinal digestion, induce the synthesis of considerable amounts of plasmatic ACE with low activity.

### 3.3 Effect of amaranth anti-hypertensive peptides on the vascular system

To determine whether the vasorelaxant effect observed after the ingestion of amaranth proteins/peptides is only due to their competitive inhibitory effect on the RAS enzymes, the contractile activity of thoracic aorta rings was determined in the presence of potassium ions and norepinephrine. These compounds are known to cause vasoconstriction through the action upon voltage- and receptor-operated channels, respectively (Rinaldi, 1990)

The contractile activity determined in the presence of potassium ions was higher in the  $G_W$ ,  $G_C$  and  $G_A$  groups (roughly 0.45 g/mg), whereas this activity was significantly lower in the animals belonging to groups  $G_{API}$ ,  $G_{VIKP}$ ,  $G_{E+VIKP}$ ,  $G_{AH}$  and  $G_E$  (0.34 to 0.29 mg/g, **Figure 4,A**). Upon treating aorta rings with physiological concentrations of norepinephrine (**Figure 4,B**), statistically differences were observed in the,  $G_{VIKP}$  and  $G_{E+VIKP}$  groups.

Potassium ions cause depolarisation of the plasma membrane and allow the opening of type L calcium channels through which calcium ions enter and cause contraction of the myofibrillar system (Rinaldi, 1990). Norepinephrine binds to  $\alpha$ -adrenergic receptors and, to a lesser extent, to  $\beta$ -adrenergic receptors to allow a rapid release of intracellular calcium and a subsequent influx of extracellular calcium, causing vascular contraction (Grand & Rinaldi, 1990; Rinaldi, Grand, & Cingolani, 1991).

The fact that no reduction in the contractile activity was found in the aorta rings treated with potassium and norepinephrine in the  $G_C$  and  $G_A$  groups was expected, since it is known that both compounds are competitive inhibitors of plasma ACE and renin, respectively (Bernstein et al., 2013). For groups  $G_{API}$ ,  $G_{AH}$ ,  $G_{VIKP}$ ,  $G_E$  and  $G_{E+VIKP}$ ,

a selective action of amaranth proteins/peptides on the membrane polarity was observed. An interaction with adrenergic receptors was only observed in the groups treated with the peptide ( $G_{VIKP}$  and  $G_{E+VIKP}$ ). These findings suggest that the amaranth peptides can exert a double effect, i.e., an overall effect on the membrane depolarisation and the opening of type L channels, and a specific effect exerted by VIKP peptides on  $\alpha$ - and  $\beta$ -adrenergic receptors. In a previous work (Fritz et al., 2011) we demonstrated that norepinephrine caused a strong contraction of aorta rings. After rinsing, and 30 min after incubation with an amaranth hydrolysate, a marked vasorelaxant effect was observed in strength vs. norepinephrine concentration curves. The vasorelaxant effect exerted by the hydrolysate remained even in the presence of L-NAME, indicating that the effect was not endothelium-dependent, contrary to the observations made by Barba de la Rosa et al. (2010) when working with amaranth glutenins. In the present work, aorta rings from rats treated with the different preparations were stimulated only with potassium and norepinephrine, i.e., in the absence of amaranth proteins. Even under these experimental conditions, a vasorelaxant effect was observed in animals treated with the synthetic peptide, which indicates that these peptides have a residual vasorelaxant effect. According to Fujita & Yoshikawa, (1999), peptides derived from food have higher affinity for the tissues and are cleared from the body more slowly than other inhibitors, such as captopril. These findings agree with our results. Fritz et al., (2011) did not observe an inotropic effect when they carried out *ex vivo* assays with isolated papillary muscles, which indicates that, *in vivo*, the hypotensive effect is not driven by diminishing the heart activity, but by a decrease in the vascular peripheral resistance.

#### 4 Conclusions

This work provides an advance in the knowledge of the properties of amaranth proteins, demonstrating that API, AH, synthetic peptides (VIKP) and API50:AH50 with or without VIKP emulsions, administered in a single dose for only 3 hours, can reduce the SBP in SHR rats from values of 200-220 mmHg to values of 140-170 mmHg. The

most active preparations were the emulsions formulated with API50:AH50 with or without VIKP that induced a reduction in SBP greater than that produced by equivalent or lower amounts, and equal measurement time after administration of amaranth hydrolysates or other proteins sources such as cod and rice. On the other hand, the SBP reduction values achieved by VIKP were similar to those obtained by administering the same dose of peptides of a genetically modified amaranth 11S globulin hydrolysate. Experiments in this work confirm the initial hypothesis, the decrease in SBP values can be attributed at least to two mechanisms of action of the amaranth peptides (**Figure 5**): their activity as competitive inhibitors of the main plasma enzymes of the RAS, i.e., ACE and renin, and, their effect as vasorelaxant agents on the vascular system. The administration of API, AH, synthetic peptides (VIKP) and API50:AH50 with or without VIKP emulsions decrease the enzymatic activity of ACE, together with an increase in plasma levels, probably to counter balance the inhibitory effect exerted by the peptides. On the other hand, the plasma levels of renin are only increased after the administration of aliskiren, API and AH, being the effect weaker than that observed for ACE. AH, VIKP and API50:AH50 emulsions with or without VIKP, have also an autocrine effect on RAS, inducing a vasorelaxation that persists even in the absence of the effector molecule. This vascular effect is achieved through a membrane depolarisation phenomenon and through direct interaction of VIKP with adrenergic receptors. This effect is endothelial NO-independent (Fritz et al., 2011). The results presented herein strongly suggest that after the gastrointestinal digestion, a variety of peptides with biological activity are released. These peptides play a role in the regulation of the blood pressure by acting on plasma ACE, plasma renin and the vascular system. Another alternative or concomitant phenomenon may be the resistance to *in vivo* hydrolysis of peptides present in the AH and in emulsions, as well as VIKP.

These results support the use of amaranth protein/peptides in the elaboration of complementary functional foods for hypertensive individuals.



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## 5 Figure Captions.

**Figure 1.** Changes in systolic blood pressure of SHR after 3 hour treatment with: Cw: water, CA: aliskiren, CC: captopril, API: amaranth isolated, AH: amaranth hydrolysate, VIKP: synthetic peptide from amaranth protein, E: O/W 20:80 emulsions, E + VIKP: O/W 20:80 emulsions + synthetic peptide.  $\Delta P$  values are presented as mean  $\pm$  SEM. (\*) mean significantly different compared to C<sub>w</sub> ( $p < 0.05$ ).

**Figure 2.(A)** Plasma renin concentration and **(B)** plasma ACE concentration at the end of the 3 hours treatment. Cw: water, CA: aliskiren, CC: captopril, API: amaranth isolated, AH: amaranth hydrolysate, VIKP: synthetic peptide from amaranth protein, E: O/W 20:80 emulsions, E + VIKP: O/W 20:80 emulsions + synthetic peptide. Data are presented as mean  $\pm$  SEM. (\*) mean significantly different compared to C<sub>w</sub> ( $p < 0.05$ ).

**Figure 3.** Plasma ACE activity. CA: aliskiren, CC: captopril, API: amaranth isolated, AH: amaranth hydrolysate, VIKP: synthetic peptide from amaranth protein, E: O/W 20:80 emulsions, E + VIKP: O/W 20:80 emulsions + synthetic peptide. This data are expressed as relative to 100% ACE activity to water control group (Cw). Data are presented as mean  $\pm$  SEM. (\*) mean significantly different compared to C<sub>w</sub> ( $p < 0.05$ ).

**Figure 4.** Effect of different samples on isolated aortic rings contracted by exposure to a high concentration of: **(A)** Potassium ion (80 mM). **(B)** Norepinephrine ( $10^{-6}$  M). CA: aliskiren, CC: captopril, API: amaranth isolated, AH: amaranth hydrolysate, VIKP: synthetic peptide from amaranth protein, E: O/W 20:80 emulsions, E + VIKP: O/W 20:80 emulsions + synthetic peptide. Data are presented as mean  $\pm$  SEM. (\*) means significantly different compared to C<sub>w</sub> ( $p < 0.05$ ).

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Table 1. IC<sub>50</sub> values for in vitro inhibition of renin and ACE of the samples tested

Sample	ACE IC <sub>50</sub>	renin IC <sub>50</sub>
Captopril	39.2±11.2 nM	N. D.
aliskiren	N. D.	0.6 nM (**)
API	0.7 ± 0.2 mg/mL(*)	> 13 mg/mL
AH	0.14±0.02 mg/ml	0.6 mg/mL
VIKP	0.175 uM	non active
(O/W) Emulsion	0.29±0.03 mg/ml	non active

N.D. not determined.

(\*) Aphalo, Martínez & Añón International Journal of Food Properties, 18:2688–2698, 2015

(\*\*) Yongfei Chen, Long Meng, Hua Shao & Feng Yu. Hypertension Research (2013) 36, 252–261

Figure 1

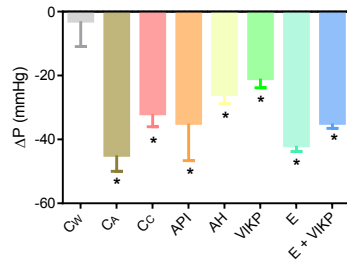


Figure 2

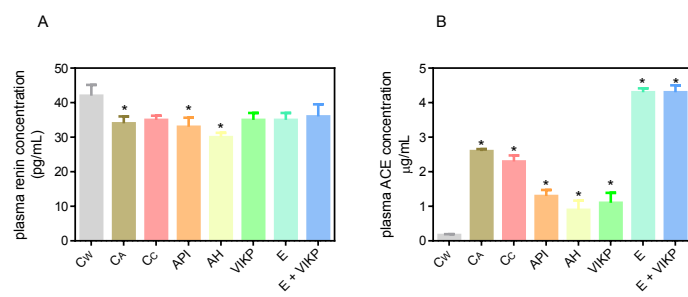


Figure 3

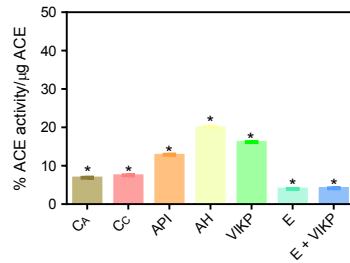
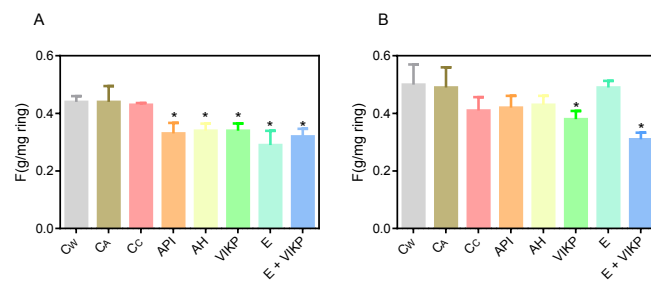
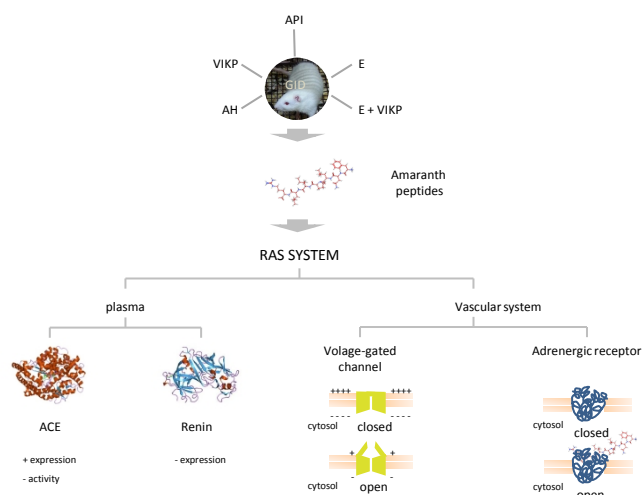


Figure 4





**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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## Highlights

- Amaranth peptides produce a very significant decrease in blood pressure in SHR rats
- These peptides have an effect on ACE and renin plasma level and ACE activity
- Amaranth peptides produce depolarization of membranes which causes vasorelaxation
- Synthetic peptide VIKP also acts on  $\beta$ -adrenergic receptors causing vasorelaxation
- Amaranth peptides act at both levels, on blood vessels and on RAS enzymes